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PATENT- G VAREMÆRKESTYRELSEN

TITLE

Stable, liquid formulation of factor VII polypeptides

FIELD OF THE INVENTION

The present invention is directed to pharmaceutical formulations containing factor VII polypeptides (FVII) and to methods for making and using such formulations. More particularly, this invention relates to such pharmaceutical formulations having increased stability in aqueous formulation.

BACKGROUND OF THE INVENTION

A variety of factors involved in the blood clotting process have been identified, including factor VII, a plasma glycoprotein. Haemostasis is initiated by the formation of a complex between tissue factor (TF) being exposed to the circulating blood following an injury to the vessel wall, and FVIIa which is present in the circulation in an amount corresponding to about 1% of the total FVII protein mass. FVII exists in plasma mainly as a single-chain zymogen, which is cleaved by FXa into its two-chain, activated form, FVIIa. Recombinant activated factor VIIa (rFVIIa) has been developed as a pro-haemostatic agent. The administration of rFVIIa offers a rapid and highly effective pro-haemostatic response in haemophilic subjects with bleedings who cannot be treated with other coagulation factor products due to antibody formation. Also bleeding in subjects with factor VII deficiency or subjects having a normal coagulation system but experiencing excessive bleeding can be treated successfully with FVIIa.

It is desirable to have finished administration forms of factor VIIa, suitable for both storage and for delivery. Ideally, the drug product is stored and administered as a liquid. Alternatively, the drug product is lyophilized, i.e., freeze-dried, and then reconstituted by adding a suitable diluent just prior to patient use. Ideally, the drug product has sufficient stability to be kept in long-term storage, i.e., more than six months.

The decision to either maintain the finished drug product as a liquid or to freeze-dry it is usually based on the stability of the protein drug in those forms. Protein stability can be affected inter alia by such factors as ionic strength, pH, temperature, repeated cycles of freeze/thaw, and exposures to shear forces. Active protein may be lost as a result of physical instabilities, including denaturation and aggregation (both soluble and insoluble aggregate formation), as well as chemical instabilities, including, for example, hydrolysis, deamidation, and oxidation, to name just a few. For a general review of stability of protein pharmaceuticals, see, for example, Manning, et al., Pharmaceutical Research 6:903-918 (1989).

While the possible occurrence of protein instabilities is widely appreciated, it is impossible to predict particular instability problems of a particular protein. Any of these instabilities can result in the formation of a protein by-product, or derivative, having lowered activity, increased toxicity, and/or increased immunogenicity. Indeed, protein precipitation may lead to thrombosis,

non-homogeneity of dosage form and amount, as well as clogged syringes. Furthermore, post-translational modifications such as, for example, gamma carboxylation of certain glutamic acid residues in the N-terminus and addition of carbohydrate side chains provide potential sites that may be susceptible to modification upon storage. Also, specific to factor VIIa, being a serin protease, fragmentation due to autocatalysis may occur. Thus, the safety and efficacy of any pharmaceutical formulation of a protein is directly related to its stability. Maintaining stability in a liquid dosage form is generally different from a lyophilized dosage form because of greatly increased potential for molecular motion and therefore increased probability of molecular interactions. Maintaining stability in a concentrated form is also different because of the propensity for aggregate formation at increased protein concentrations.

When developing a liquid formulation, many factors are taken into consideration. Short-term, i.e., less than six months, liquid stability generally depends on avoiding gross structural changes, such as denaturation and aggregation. These processes are described in the literature for a number of proteins, and many examples of stabilizing agents exist. It is well known that an agent effective at stabilizing one protein actually acts to destabilize another. Once the protein has been stabilized against gross structural changes, developing a liquid formulation for long-term stability (e.g., greater than six months) depends on further stabilizing the protein from types of degradation specific to that protein. More specific types of degradation may include, for example, disulfide bond scrambling, oxidation of certain residues, deamidation, cyclization. Although it is not always possible to pinpoint the individual degradation species, assays are developed to monitor subtle changes so as to monitor the ability of specific excipients to uniquely stabilize the protein of interest.

In addition to stability considerations, one generally selects excipients, which are approved by various worldwide medical regulatory agencies. It is highly desirable that the formulation is approximately isotonic and that the pH of the formulation is in a physiologically suitable range upon injection/infusion, otherwise pain and discomfort for the patient may result. The choice and amount of buffer used is important to achieve the desired pH range. The choice and amount of agents used to modify tonicity is important to assure the best possible patient convenience when the formulations are administered.

For a general review of protein formulations, see, for example, Cleland et al.: The development of stable protein formulations: A closer look at protein aggregation, deamidation and oxidation, Critical Reviews in Therapeutic Drug Carrier Systems 1993, 10(4): 307-377; and Wang et al., Parenteral formulations of proteins and peptides: Stability and stabilizers, Journal of Parenteral Science and Technology 1988 (Supplement), 42 (2S).

Other publications of interest regarding stabilization of proteins are as follows.

U.S. 20010031721 A1 (American Home Products) concerns highly concentrated, lyophilised, and liquid factor IX formulations.

U.S. 5,770,700 (Genetics Institute) concerns liquid factor IX formulations.

WO 97/19687 (American Red Cross) concerns liquid formulations of plasma proteins, in particular factor VIII and factor IX.

U.S. 4,297,344 discloses stabilization of coagulation factors II and VIII, antithrombin III, and plasminogen against heat by adding selected amino acids such as glycine, alanine, hydroxyproline, glutamine, and aminobutyric acid, and a carbohydrate such as a monosaccharide, an oligosaccharide, or a sugar alcohol.

Traditionally, large labile proteins, such as factor VII, are administered intravenously, either prophylactically or in response to bleeding episodes. Given intravenously, the protein is directly available in the blood stream. Unfortunately, there can be side effects associated with repeated injections, including occlusion and/or fibrin formation, especially in the elderly. Moreover, where the patient's veins are particularly small, e.g., in small children, it can be difficult to achieve the requisite therapeutic dose.

Factor VIIa undergoes several degradative pathways, especially dimerisation, oxidation, and autolytic cleavage (clipping of the peptide backbone). Furthermore, precipitation may occur. Many of these reactions can be slowed significantly by removal of water from the protein. However, the development of an aqueous formulation for factor VIIa has the advantages of eliminating reconstitution errors, thereby increasing dosing accuracy, as well as simplifying the use of the product clinically, thereby increasing patient compliance. Ideally, formulations of factor VIIa should be stable for more than 6 months over a wide range of protein concentrations. This allows for flexibility in methods of administration. Generally, more highly concentrated forms allow for the administration of lower volumes, which is highly desirable from the patients' point of view. Liquid formulations can have many advantages over freeze-dried products with regard to ease of administration and use.

Today, the only commercially available, recombinantly-made FVII polypeptide formulation is a freeze-dried factor FVIIa product which is reconstituted before use, and is limited to a low factor VIIa concentration, e.g., about 0.6 mg/ml.. The recombinant human factor VIIa formulation known in the art is a lyophilised preparation requiring reconstitution. Per vial (1.2 mg), NovoSeven® (Novo Nordisk A/S, Denmark) consists of 1.2 mg recombinant human factor VIIa, 5.84 mg NaCl, 2.94 mg CaCl2, 2 H2O, 2.64 mg GlyGly, 0.14 mg polysorbate 80, 60.0 mg mannitol, reconstituted to pH 5.5 by 2.0 ml water for injection (WFI). When reconstituted, the protein solution is stable for 24 hours. Thus, no liquid or highly concentrated factor VII products are currently commercially available.

Accordingly, there is a need in the art for methods for improving stability of factor VII polypeptides (including factor VIIa), increasing the concentration, maintaining activity levels, and providing liquid formulations suitable for prolonged storage for more than 6 months at 2 to 8°C. Thus, it is an objective of this invention to provide an aqueous factor VII polypeptide formulation which provides acceptable control of degradation products, is stable to agitation (which induces precipitation), and is resistant to microbial contamination (which allows multiple-use packaging).

SUMMARY OF THE INVENTION

We have discovered that factor VII or analogu s thereof ("factor VII polypeptides"), when formulated in aqueous solution together with a buffer, a calcium salt, and a tonicity modifier, are physically and chemically stable in the pH range from about 4 to about 7. Such formulations are physically and chemically stable within a given shelf life period at the recommended storage temperature (typically 0.5 -3 years at 2-8°C). Furthermore, the present formulations are physically and chemically stable during in-use (typically 1 month at accelerated temperatures, e.g., 25°C or 37°C).

In one aspect, the present invention provides a stable, aqueous liquid pharmaceutical formulation, comprising a factor VII polypeptide, a calcium salt, a buffer, and a tonicity modifier, wherein the formulation has a pH of from about 4.0 to about 7.0. In one embodiment, the formulation has a pH of from about 5.0 to about 6.0.

In different embodiments, the tonicity modifier is an amino acid; a neutral salt; a mono- or disaccharide; a polysaccharide; a sugar alcohol, or a mixture of at least two of these modifiers. In other embodiments, the tonicity modifier is present at a concentration of from about 1 to about 500 mM; from about 1 to about 300 mM; from about 10 to about 200 mM; or from about 20 to about 150 mM. In one embodiment, the tonicity modifier is NaCl.

In one embodiment, the buffer is selected from the list of citrate, acetate, histidine, malate, phosphate, tartaric acid, succinic acid, MES (2-N-morpholino-ethanesulphonic acid), HEPES (4-(2-hydroxy-ethyl)-piperazine-1-ethane-sulphonic acid), imidazol, TRIS (tris (hydroxymethyl) aminomethane), lactate, glutamate, or a mixture of at least two of these.

In one embodiment, the concentration of the buffer is from 1 mM to 50 mM. In one embodiment, the concentration of the buffer is about 10 mM.

In one embodiment, the buffer is citrate. In another embodiment, the buffer is a mixture of at least two of citrate, acetate, histidine, malate, phosphate, tartaric acid, succinic acid, MES, HEPES, imidazol, TRIS, lactate, and glutamate. In another embodiment, the buffer is a mixture of citrate and at least one of acetate, histidine, malate, phosphate, tartaric acid, succinic acid, MES, HEPES, imidazol, TRIS, lactate, and glutamate. In one embodiment of the buffer being a mixture of citrate and at least one other component, the citrate is present at a concentration of from 1 mM to10 mM.

In one embodiment, the calcium salt is present in a concentration of 5 mM to 100 mM. In another embodiment, the calcium salt is present in a concentration of 10 mM to 50 mM. In one embodiment, the calcium salt is CaCl2, calcium lactate, calciumgluconate, calciumacetate, or a mixture of at least two of these.

In one embodiment, the formulation further comprises an antioxidant such as ascorbic acid, cysteine or methionine including homologues like homocysteine, isomers like D-

methionine, or peptide derivatives, e.g. gluthatione, or methionine- analogues. In a preferred embodiment, the antioxidant is L-methionine.

In one embodiment, the formulation also comprises a non-ionic surfactant. In one embodiment, the non-ionic surfactant is present in an amount of from 0.01 to 1% by weight. In one embodiment, the non-ionic surfactant is a polysorbate or a poloxamer or a polyoxyethylene alkyl ether. In one embodiment, the poloxamer is poloxamer 188 or poloxamer 407, or the polysorbate is polysorbate 20 or polysorbate 80, or the polyoxyethylene alkyl ether is BriJ35®.

In one embodiment, the formulation also comprises glycylglycine or an amino acid or a small peptide, e.g., a peptide comprising from 2 to 5 amino acid residues.

In one embodiment, the formulation further comprises a preservative. In one embodiment, the preservative is selected from the list of phenol, benzyl alcohol, orto-cresol, meta-cresol, para-cresol, methyl paraben, propyl paraben, benzalconium chloride and benzaethonium chloride.

In one embodiment the formulation is isotonic. In one embodiment, the formulation is sterile. In one embodiment, the formulation is a liquid aqueous formulation. In one embodiment, the formulation is stable during storage for at least 6 months at 2-8°C. In one embodiment, the formulation is chemically and physically stable for at least 6 months when stored at a temperature of from 2 to 8°C. In a further embodiment, the formulation is chemically and physically stable for at least 6 months when stored at a temperature of from 2 to 8°C, and, following storage, additionally stable for at least 4 weeks when kept at a temperature of from 25 to 37°C.

In one embodiment, the factor VII polypeptide is human factor VIIa. In one embodiment, the factor VII polypeptide is a factor VII sequence variant. In one embodiment, the factor VII polypeptide has a glycosylation different from wild-type human factor VII. In one embodiment, the factor VII polypeptide is factor VII chemically inactivated by reaction with D-phe-phe-Arg chloromethylketone (FFR-FVIIa).

In one embodiment, the factor VII polypeptide is present in a concentration of from 0.1 mg/ml to 10 mg/ml. In another embodiment, the factor VII polypeptide is present in a concentration of from 0.5 mg/ml to 5.0 mg/ml. In another embodiment, the factor VII polypeptide is present in a concentration of from 0.5 mg/ml to 4.0 mg/ml.

In a further aspect, the present invention relates to a method for preparing a stable, liquid pharmaceutical formulation of a factor VII polypeptide, comprising preparing a formulation containing said factor VII polypeptide, a calcium salt, a buffer, and a tonicity modifier, wherein the formulation has a pH of from 4.0 to 7.0.

In a further aspect, the present invention relates to a method for treating a factor VII-responsive syndrome, the method comprising administering to a subject in need thereof, under conditions that result in a decrease in bleeding and/or an increase in blood clotting, an effective amount of a stable, aqueous liquid pharmaceutical formulation comprising a factor VII polypeptide, a calcium salt, a, and a tonicity modifier, wherein the formulation has a pH from 4.0 to 7.0.

In different embodiments, the syndrome is selected from the group consisting of haemophilia A, haemophilia B, Factor XI deficiency, Factor VII deficiency, thrombocytopenia, von Willebrand's disease, presence of a clotting factor inhibitor, surgery, trauma, and anticoagulant therapy.

In a further aspect, the present invention relates to a method for preventing an unwanted bleeding, the method comprising administering to a subject in need thereof, under conditions that result in a decrease in bleeding and/or an increase in blood clotting, an effective amount of a stable, aqueous liquid pharmaceutical formulation comprising a factor VII polypeptide, a calcium salt, a, and a tonicity modifier, wherein the formulation has a pH from 4.0 to 7.0.

In a further aspect, the present invention relates to a method for preventing unwanted blood clotting, the method comprising administering to a subject in need thereof, under conditions effective for inhibiting coagulation, an effective amount of a stable, aqueous liquid pharmaceutical formulation comprising a factor VII polypeptide, a calcium salt, a, and a tonicity modifier, wherein the formulation has a pH from 4.0 to 7.0.

In different embodiments, the unwanted blood clotting is associated with a condition selected from the group consisting of: angioplasty, deep vein thrombosis, pulmonary embolism, stroke, disseminated intravascular coagulation (DIC), fibrin deposition in lungs and kidneys associated with gram-negative endotoxemia, and myocardial infarction.

In a further aspect, the present invention relates to a method for preventing tissue factor mediated reactions, the method comprising administering to a subject in need thereof, under conditions effective for inhibiting coagulation, an effective amount of a stable, aqueous liquid pharmaceutical formulation comprising a factor VII polypeptide, a calcium salt, a, and a tonicity modifier, wherein the formulation has a pH from 4.0 to 7.0.

In different embodiments, the tissue factor mediated reactions are associated with a condition selected from the group consisting of SIRS, ARDS, MOF,HUS, and TTP.

DETAILED DESCRIPTION OF THE INVENTION

The formulations according to the present invention are useful as storage-stable, and preferably ready-to-use, medicaments for administration. These formulations are stable for at least six months, and preferably up to 36 months; when stored at temperatures ranging from 2° to 8° C. The formulations may also, normally when in use, be stored for at least one month at temperatures ranging from 2° to 40° C.

"Stable" is intended to mean that the formulation, after storage for 6 months at 2 to 8°C retains at least 50% of its initial biological activity as measured by a one-stage clot assay essentially as described in WO 92/15686. Briefly, the sample to be tested is diluted in 50 mM Tris (pH 7.5), 0.1% BSA and 100 µl is incubated with 100 µl of Factor VII deficient plasma and 200 µl of thromboplastin C containing 10 mM Ca^{2+}. Clotting times are measured and compared to a standard curve using a pool of citrated normal human plasma in serial dilution. Preferably, the

stable formulation retains at least 80% of its initial activity after storage for 6 months at 2 to 8°C.

The term"physically stable" is intended to designate a formulation which remains visually clear. Physical stability of the formulations is evaluated by means of visual inspection and turbidity after storage of the formulation at different temperatures in top filled glass cartridges for various time periods. Visual inspection of the formulations is performed in a sharp focused light with a dark background. The turbidity of the formulation is characterized by a visual score ranking the degree of turbidity, e.g., from 0 to 3 (a formulation showing no turbidity corresponds to a visual score 0, and a formulation showing visual turbidity in daylight corresponds to visual score 3). A formulation is classified physical unstable, when it shows visual turbidity in daylight.

The term "chemically stable" is intended to designate a formulation which retains at least 50% of its initial biological activity after storage for 6 months at 2 to 8°C, as measured by a one-stage clot assay essentially as described in WO 92/15686.

The compositions comprise factor VII polypeptides, calcium ions, tonicity modifiers, buffering agents, and, optionally, other excipients, which further stabilize the factor VII polypeptides. The factor VII polypeptides concentration ranges from about 0.1 to about 10 mg/mL. As used herein, the term "tonicity modifier" includes agents, which contribute to the osmolality of the solution. Tonicity modifiers include, but are not limited to, amino acids; small peptides (e.g., having from 2 to 5 amino acid residues); neutral salts; mono- or disaccharides; polysaccharides; sugar alcohols, or a mixture of at least two of said modifiers. Examples of tonicity modifiers include, but are not limited to, sodium chloride, potassium chloride, sodium citrate, sucrose, glucose, and mannitol. The concentration of the modifiers is adjusted to near isotonicity, depending on the other ingredients present in the formulation. Normally, the modifiers are present at a concentration of from about 1 to about 500 mM; from about 1 to about 300 mM; from about 10 to about 200 mM; or from about 20 to about 150 mM, depending on the other ingredients present. Neutral salts such as, e.g., sodium chloride or potassium chloride may be used. By "neutral salt" is meant a salt that is neither an acid nor a base when dissolved in aqueous solution.

The term "buffering agent" encompasses those agents, which maintain the solution pH in an acceptable range from 4.0 to 7.0, 5.0 to 7.0, 5.0 to 6.5, 5.5 to 6.5, 5.0 to 6.0, 5.2 to 5.7, or about 5.5, and may include, but are not limited to, citrate (sodium or potassium), acetate (ammonium, sodium or calcium), histidine (L-histidine), malate, phosphate (sodium or potassium), tartaric acid, succinic acid, MES, HEPES, imidazol, TRIS, lactate, and glutamate. The buffer concentration range is chosen to maintain the preferred pH of the solution. The buffering agent may also be a mixture of at least two buffering agents, wherein the mixture is able to provide a pH value in the specified range. In one embodiment, the buffering agent is selected from the list of citrat (sodium or potassium), acetate (ammonium, sodium or calcium), histidine (L-

histidine), malate, phosphate (sodium or potassium), tartaric acid, succinic acid, MES, HEPES, imidazol, TRIS, lactate, and glutamate, or a mixture of at least two of said buffering agents. In one embodiment, the buffering agent is a mixture of two of said agents. In one embodiment, the buffer is a mixture of citrate and at least one of the buffers acetate (ammonium, sodium or calcium), histidine (L-histidine), malate, phosphate (sodium or potassium), tartaric acid, succinic acid, MES, HEPES, imidazol, TRIS, lactate, and glutamate. In alternative embodiments, the buffer concentration is in the range of from about 1 mM to 100 mM; from 1 mM to about 50 mM; from about 1 mM to about 25 mM; from about 2 mM to about 20 mM; or about 10 mM.

Optionally, the compositions may also contain a surfactant or detergent. "Surfactants" or "detergents" generally include those agents which protect the protein from air/solution interface induced stresses and solution/surface induced stresses (e.g., resulting in protein aggregation). The detergent is preferably a non-ionic detergent including, but not limited to polysorbates(e.g. Tween®), such as polysorbate 20 or 80; polyoxyethylene alkyl ethers or poloxamers, such as poloxamer 188 or 407, (e.g., Pluronic® polyols) and other ethylene/polypropylene block polymers, or polyethyleneglycol (PEG) such as PEG8000. The amount of surfactant present ranges from about 0.005 to 1%, with about 0.005 to 0.1% and about 0.005 to 0.02% preferred. Optionally, relatively high concentrations, e.g., up to 0.5%, are suitable for maintaining protein stability; however, the levels used in actual practice are customarily limited by clinical practice

Optionally, the formulation may include an antioxidant. Antioxidants include, but are not limited to, ascorbic acid, cysteine, homocysteine, cystine, cysstathionine, methionine, gluthatione, and other peptides containing cysteine or methionine; methionine, in particular L-methionine, is preferred. The antioxidant is included at a concentration of 0.1 to 2 mg/ml.

A preservative may be included in the formulation to retard microbial growth and thereby allow "multiple use" packaging of the FVII polypeptides. Preservatives include phenol, benzyl alcohol, orto-cresol, meta-cresol, para-cresol, methyl paraben, propyl paraben, benzalconium chloride, and benzethonium chloride. The preservative is normally included at a concentration of 0.1 to 2 mg/ml

Optionally, the formulation may include a stabilizer. Stabilizers include, but are not limited to, amino acids and small peptides (e.g., having from 2 to 5 amino acid residues). Examples of stabilizers include glycylglycine. The stabilizers are preferably present in an amount of from 1 mg/ml to 50 mg/ml.

It will be understood that the above quantities are somewhat flexible within ranges, as set forth in more detail above, and that the materials are interchangeable within the component categories. That is, polysorbate 80, or a poloxamer, may be substituted for polysorbate 20, an acetate buffer could instead be employed, and alternative preservatives could be used. In addition, more than one buffering agent, preservative, neutral salt, or non-ionic surfactant may be used. Preferably, the formulation is isotonic and sterile.

As used herein, amounts specified are understood to be +about 10%, e.g., about 50 mM includes 50 mM +/- 5 mM; e.g., 4% includes 4% +/- 0.4%, etc.

Percentages are weight/weight both when referring to solids dissolved in solution and liquids mixed into solutions. For example, for Tween, it is the weight of 100% stock/weight of solution.

The term "isotonic" means "isotonic with serum," 300 +/- 50 milliosmol, and is meant to be a measure of osmolality of the solution prior to administration. Maintaining physiological osmolality is important when the dosage formulations are meant to be injectable without prior dilution.

The term "an effective amount" is the effective dose to be determined by a qualified practitioner, who may titrate dosages to achieve the desired response. Factors for consideration of dose will include potency, bioavailability, desired pharmacokinetic/pharmacodynamic profiles, condition of treatment, patient-related factors (e.g. weight, health, age, etc.), presence of co-administered medications (e.g., anticoagulants), time of administration, or other factors known to a medical practitioner.

The term "treatment" is defined as the management and care of a subject, e.g. a mammal, in particular a human, for the purpose of combating the disease, condition, or disorder and includes the administration of a factor VII polypeptide to prevent the onset of the symptoms or complications, or alleviating the symptoms or complications, or eliminating the disease, condition, or disorder. Pharmaceutical compositions according to the present invention containing a factor VII polypeptide may be administered parenterally to subjects in need of such a treatment. Parenteral administration may be performed by subcutaneous, intramuscular or intravenous injection by means of a syringe, optionally a pen-like syringe. Alternatively, parenteral administration can be performed by means of an infusion pump.

A pharmaceutical formulation is found to be physically unstable when it exhibits turbidity. Physical stability of the formulations is evaluated by means of visual inspection and spectrofotometric measurement of solution turbidity. Visual inspection of the formulations is performed in a sharp focused light with a dark background.

Factor VIIa concentration is conveniently expressed as mg/mL or as IU/mL, with 1 mg usually representing 43000 – 56000 IU or more.

The term "pharmaceutically effective amount" of factor VII polypeptides refers to that amount that provides therapeutic effect in an administration regimen. The compositions of the present invention are prepared containing amounts of factor VII polypeptides at least about 0.1 mg/ml, upwards of about 0.6 mg/ml, upwards of about 1 mg/ml, or from about 0.6 mg/ml to about 10 mg/ml. For use of these compositions in administration to human subjects suffering from coagulation disorders or bleedings, for example, these compositions contain from about 0.6 mg/ml to about 10 mg/ml, corresponding to the currently contemplated dosage regimen for the

intended treatment. The dose range is not critical to the invention, and may be varied by the clinician.

Methods of use:

The preparations of the present invention may be used to treat any Factor VII-responsive syndrome, such as, e.g., bleeding disorders, including, without limitation, those caused by clotting factor deficiencies (e.g., haemophilia A and B or deficiency of coagulation factors XI or VII); by thrombocytopenia or von Willebrand's disease, or by clotting factor inhibitors, or excessive bleeding from any cause. The preparations may also be administered to patients in association with surgery or other trauma or to patients receiving anticoagulant therapy.

Preparations according to the invention, comprising Factor VII-related polypeptides, which have substantially reduced bioactivity relative to wild-type Factor VII, may be used as anticoagulants, such as, e.g., in patients undergoing angioplasty or other surgical procedures that may increase the risk of thrombosis or occlusion of blood vessels as occurs, e.g., in restenosis. Other medical indications for which anticoagulants are prescribed include, without limitation, deep vein thrombosis, pulmonary embolism, stroke, disseminated intravascular coagulation (DIC), fibrin deposition in lungs and kidneys associated with gram-negative endotoxemia, myocardial infarction; Acute Respiratory Distress Syndrome (ARDS), Systemic Inflammatory Response Syndrome (SIRS), Hemolytic Uremic Syndrome (HUS), MOF, and TTP.

Factor VII polypeptides to be formulated according to the present invention:

The terms "human factor VII" or "FVII" denote human factor VII produced by methods including natural source extraction and purification, and by recombinant cell culture systems. Its sequence and characteristics are set forth, for example, in US Patent No. 4,784,950. The terms likewise cover biologically active human factor VII equivalents, e.g., differing in one or more amino acid(s) in the overall sequence. Furthermore, the terms used in this application are intended to cover substitution, deletion and insertion amino acid variants of factor VII or post-translational modifications. As used herein, "Factor VII polypeptide" encompasses, without limitation, Factor VII, as well as equivalents of Factor VII. Factor VII equivalents include, without limitation, Factor VII polypeptides that have either been chemically modified relative to human Factor VII and/or contain one or more amino acid sequence alterations relative to human Factor VII (i.e., Factor VII variants), and/or contain truncated amino acid sequences relative to human Factor VII (i.e., Factor VII fragments). Such equivalents may exhibit different properties relative to human Factor VII, including stability, phospholipid binding, altered specific activity, and the like. The term "Factor VII" is intended to encompass Factor VII polypeptides in their uncleaved (zymogen) form, as well as those that have been proteolytically processed to yield their respective bioactive forms, which may be designated Factor VIIa. Typically, Factor VII is cleaved between residues 152 and 153 to yield Factor VIIa. The term "Factor VII" is also intended to encompass,

without limitation, polypeptides having the amino acid sequence 1-406 of wild-type human Factor VII (as disclosed in U.S. Pat nt No. 4,784,950), as well as wild-type Factor VII derived from other species, such as, e.g., bovine, porcine, canine, murine, and salmon Factor VII. It further encompasses natural allelic variations of Factor VII that may exist and occur from one individual to another. Also, degree and location of glycosylation or other post-translation modifications may vary depending on the chosen host cells and the nature of the host cellular environment.
As used herein, "Factor VII equivalent" encompasses, without limitation, equivalents of Factor VII exhibiting substantially the same or improved biological activity relative to wild-type human Factor VII, as well as equivalents, in which the Factor VIIa biological activity has been substantially modified or reduced relative to the activity of wild-type human Factor VIIa. These polypeptides include, without limitation, Factor VII or Factor VIIa that has been chemically modified and Factor VII variants into which specific amino acid sequence alterations have been introduced that modify or disrupt the bioactivity of the polypeptide.

It further encompasses polypeptides with a slightly modified amino acid sequence, for instance, polypeptides having a modified N-terminal end including N-terminal amino acid deletions or additions, and/or polypeptides that have been chemically modified relative to human Factor VIIa.

Equivalents, including variants of Factor VII, whether exhibiting substantially the same or better bioactivity than wild-type Factor VII, or, alternatively, exhibiting substantially modified or reduced bioactivity relative to wild-type Factor VII, include, without limitation, polypeptides having an amino acid sequence that differs from the sequence of wild-type Factor VII by insertion, deletion, or substitution of one or more amino acids.

Factor VII equivalents, including variants, encompass those that exhibit at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 100%, at least about 110%, at least about 120%, and at least about 130%, of the specific activity of wild-type Factor VIIa that has been produced in the same cell type, when tested in one or more of a clotting assay, proteolysis assay, or TF binding assay as described above.

Factor VII equivalents, including variants, having substantially the same or improved biological activity relative to wild-type Factor VIIa encompass those that exhibit at least about 25%, preferably at least about 50%, more preferably at least about 75%, more preferably at least about 100%, more preferably at least about 110%, more preferably at least about 120%, and most preferably at least about 130% of the specific activity of wild-type Factor VIIa that has been produced in the same cell type, when tested in one or more of a clotting assay, proteolysis assay, or TF binding assay as described above.

Factor VII equivalents, including variants, having substantially reduced biological activity relative to wild-type Factor VIIa are those that exhibit less than about 25%, preferably less than about 10%, more preferably less than about 5% and most preferably less than about 1% of

the specific activity of wild-type Factor VIIa that has been produced in the same cell type when tested in one or more of a clotting assay, proteolysis assay, or TF binding assay as described above. Factor VII variants having a substantially modified biological activity relative to wild-type Factor VII include, without limitation, Factor VII variants that exhibit TF-independent Factor X proteolytic activity and those that bind TF but do not cleave Factor X.

In some embodiments the Factor VII polypeptides are Factor VII equivalents, in particular variants, wherein the ratio between the activity of said Factor VII polypeptide and the activity of native human Factor VIIa (wild-type FVIIa) is at least about 1.25 when tested in the "In Vitro Hydrolysis Assay" (see "Assays", below); in other embodiments, the ratio is at least about 2.0; in further embodiments, the ratio is at least about 4.0. In some embodiments of the invention, the factor VII polypeptides are Factor VII equivalents, in particular variants, wherein the ratio between the activity of said Factor VII polypeptide and the activity of native human Factor VIIa (wild-type FVIIa) is at least about 1.25 when tested in the "In Vitro Proteolysis Assay" (see "Assays", below); in other embodiments, the ratio is at least about 2.0; in further embodiments, the ratio is at least about 4.0; in further embodiments, the ratio is at least about 8.0.

In some embodiments, the Factor VII polypeptide is human Factor VII, as disclosed, e.g., in U.S. Patent No. 4,784,950 (wild-type Factor VII). In some embodiments, the Factor VII polypeptide is human Factor VIIa. In one series of embodiments, the Factor VII polypeptides are Factor VII equivalents that exhibits at least about 10%, preferably at least about 30%, more preferably at least about 50%, and most preferably at least about 70%, of the specific biological activity of human Factor VIIa. In one series of embodiments, Factor VII polypeptides include polypeptides that exhibit at least about 90%, preferably at least about 100%, preferably at least about 120%, more preferably at least about 140%, and most preferably at least about 160%, of the specific biological activity of human Factor VIIa.

In some embodiments, the Factor VII polypeptides have an amino acid sequence that differs from the sequence of wild-type Factor VII by insertion, deletion, or substitution of one or more amino acids.

In one series of embodiments, Factor VII polypeptides include polypeptides that exhibit at least about 70 %, preferably at least about 80 %, more preferably at least about 90 %, and most preferable at least about 95 %, of identity with the sequence of wild-type Factor VII as disclosed in U.S. Patent No. 4,784,950.

Non-limiting examples of Factor VII variants having substantially the same or improved biological activity as wild-type Factor VII include S52A-FVII, S60A-FVII (Iino et al., Arch. Biochem. Biophys. 352: 182-192, 1998); L305V-FVII, L305V/M306D/D309S-FVII, L305I-FVII, L305T-FVII, F374P-FVII, V158T/M298Q-FVII, V158D/E296V/M298Q-FVII, K337A-FVII, M298Q-FVII, V158D/M298Q-FVII, L305V/K337A-FVII, V158D/E296V/M298Q/L305V-FVII, V158D/E296V/M298Q/K337A-FVII, V158D/E296V/M298Q/L305V/K337A-FVII, K157A-FVII, E296V-FVII, E296V/M298Q-FVII, V158D/E296V-FVII, V158D/M298K-FVII, and S336G-FVII; FVIIa variants exhibiting increased TF-

independent activity as disclosed in [LISTE OVER FVII VARIANT ANSØGNINGER]; FVIIa variants exhibiting increased proteolytic stability as disclosed in U.S. Patent No. 5,580,560; Factor VIIa that has been proteolytically cleaved between residues 290 and 291 or between residues 315 and 316 (Mollerup et al., Biotechnol. Bioeng. 48:501-505, 1995); and oxidized forms of Factor VIIa (Kornfelt et al., Arch. Biochem. Biophys. 363:43-54, 1999). Non-limiting examples of Factor VII variants having substantially reduced or modified biological activity relative to wild-type Factor VII include R152E-FVIIa (Wildgoose et al., Biochem 29:3413-3420, 1990), S344A-FVIIa (Kazama et al., J. Biol. Chem. 270:66-72, 1995), FFR-FVIIa (Holst et al., Eur. J. Vasc. Endovasc. Surg. 15:515-520, 1998), and Factor VIIa lacking the Gla domain, (Nicolaisen et al., FEBS Letts. 317:245-249, 1993). Non-limiting examples of chemically modified Factor VII polypeptides and sequence variants are described, e.g., in U.S. Patent No. 5,997,864.

Biological activity of factor VII polypeptides:

The biological activity of Factor VIIa in blood clotting derives from its ability to (i) bind to tissue factor (TF) and (ii) catalyze the proteolytic cleavage of Factor IX or Factor X to produce activated Factor IX or X (Factor IXa or Xa, respectively).

For purposes of the invention, biological activity of Factor VII polypeptides ("Factor VII biological activity") may be quantified by measuring the ability of a preparation to promote blood clotting using Factor VII-deficient plasma and thromboplastin, as described, e.g., in U.S. Patent No. 5,997,864 or WO 92/15686. In this assay, biological activity is expressed as the reduction in clotting time relative to a control sample and is converted to "Factor VII units" by comparison with a pooled human serum standard containing 1 unit/ml Factor VII activity. Alternatively, Factor VIIa biological activity may be quantified by

- Measuring the ability of Factor VIIa or a Factor VIIa equivalent to produce activated Factor X (Factor Xa) in a system comprising TF embedded in a lipid membrane and Factor X. (Persson et al., J. Biol. Chem. 272:19919-19924, 1997);
- Measuring Factor X hydrolysis in an aqueous system ("In Vitro Proteolysis Assay", see below);
- Measuring the physical binding of Factor VIIa or a Factor VIIa equivalent to TF using an instrument based on surface plasmon resonance (Persson, FEBS Letts. 413:359-363, 1997); and
- Measuring hydrolysis of a synthetic substrate by Factor VIIa and/or a Factor VIIa equivalent ("In Vitro Hydrolysis Assay", see below); and
- Measuring generation of thrombin in a TF-independent in vitro system.

Assays suitable for determining biological activity of factor VII polypeptides:

Factor VII polypeptides useful in accordance with the present invention may be selected by suitable assays that can be performed as simple preliminary in vitro tests. Thus, the present

specification discloses a simple test (entitled "In Vitro Hydrolysis Assay") for the activity of Factor VII polypeptides.

In Vitro Hydrolysis Assay

Native (wild-type) factor VIIa and Factor VII polypeptide (both hereafter referred to as "factor VIIa") may be assayed for specific activities. They may also be assayed in parallel to directly compare their specific activities. The assay is carried out in a microtiter plate (MaxiSorp, Nunc, Denmark). The chromogenic substrate D-Ile-Pro-Arg-p-nitroanilide (S-2288, Chromogenix, Sweden), final concentration 1 mM, is added to factor VIIa (final concentration 100 nM) in 50 mM Hepes, pH 7.4, containing 0.1 M NaCl, 5 mM CaCl2 and 1 mg/ml bovine serum albumin. The absorbance at 405 nm is measured continuously in a SpectraMax™ 340 plate reader (Molecular Devices, USA). The absorbance developed during a 20-minute incubation, after subtraction of the absorbance in a blank well containing no enzyme, is used to calculate the ratio between the activities of Factor VII polypeptide and wild-type factor VIIa:

Ratio = (A405 nm Factor VII polypeptide)/(A405 nm factor VIIa wild-type).

Based thereon, Factor VII polypeptides with an activity lower than, comparable to, or higher than native factor VIIa may be identified, such as, for example, Factor VII polypeptides where the ratio between the activity of the Factor VII polypeptide and the activity of native factor VII (wild-type FVII) is about, versus above 1.0.

The activity of the Factor VII polypeptides may also be measured using a physiological substrate such as factor X ("In Vitro Proteolysis Assay"), suitably at a concentration of 100-1000 nM, where the factor Xa generated is measured after the addition of a suitable chromogenic substrate (eg. S-2765). In addition, the activity assay may be run at physiological temperature.

In Vitro Proteolysis Assay

Native (wild-type) Factor VIIa and Factor VII polypeptide (both hereafter referred to as "Factor VIIa") are assayed in parallel to directly compare their specific activities. The assay is carried out in a microtiter plate (MaxiSorp, Nunc, Denmark). Factor VIIa (10 nM) and Factor X (0.8 microM) in 100 microL 50 mM Hepes, pH 7.4, containing 0.1 M NaCl, 5 mM CaCl2 and 1 mg/ml bovine serum albumin, are incubated for 15 min. Factor X cleavage is then stopped by the addition of 50 microL 50 mM Hepes, pH 7.4, containing 0.1 M NaCl, 20 mM EDTA and 1 mg/ml bovine serum albumin. The amount of Factor Xa generated is measured by addition of the chromogenic substrate Z-D-Arg-Gly-Arg-p-nitroanilide (S-2765, Chromogenix, Sweden), final concentration 0.5 mM. The absorbance at 405 nm is measured continuously in a SpectraMax™ 340 plate reader (Molecular Devices, USA). The absorbance developed during 10 minutes, after subtraction of the absorbance in a blank well containing no FVIIa, is used to calculate the ratio between the proteolytic activities of Factor VII polypeptide and wild-type Factor VIIa:

Ratio = (A405 nm Factor VII polypeptide)/(A405 nm Factor VIIa wild-type).

Based thereon, Factor VII polypeptide with an activity lower than, comparable to, or higher than native factor VIIa may be identified, such as, for example, Factor VII polypeptides

where the ratio between the activity of the Factor VII polypeptide and the activity of native factor VII (wild-type FVII) is about, versus above 1.0.

The ability of factor VIIa or Factor VII polypeptides to generate thrombin can also be measured in an assay comprising all relevant coagulation factors and inhibitors at physiological concentrations (minus factor VIII when mimicking hemophilia A conditions) and activated platelets (as described on p. 543 in Monroe et al. (1997) Brit. J. Haematol. 99, 542-547, which is hereby incorporated as reference).

The activity of the Factor VII polypeptides may also be measured using a one-stage clot assay essentially as described in WO 92/15686 or US 5,997,864. Briefly, the sample to be tested is diluted in 50 mM Tris (pH 7.5), 0.1% BSA and 100 µl is incubated with 100 µl of Factor VII deficient plasma and 200 µl of thromboplastin C containing 10 mM Ca^{2+}. Clotting times are measured and compared to a standard curve using a pool of citrated normal human plasma in serial dilution.

Preparation and purification of factor VII polypeptides:

Human purified Factor VIIa suitable for use in the present invention is preferably made by DNA recombinant technology, e.g. as described by Hagen et al., Proc.Natl.Acad.Sci. USA 83: 2412-2416, 1986, or as described in European Patent No. 200.421 (ZymoGenetics, Inc.).

Factor VII may also be produced by the methods described by Broze and Majerus, J.Biol.Chem. 255 (4): 1242-1247, 1980 and Hedner and Kisiel, J.Clin.Invest. 71: 1836-1841, 1983. These methods yield Factor VII without detectable amounts of other blood coagulation factors. An even further purified Factor VII preparation may be obtained by including an additional gel filtration as the final purification step. Factor VII is then converted into activated factor VIIa by known means, e.g. by several different plasma proteins, such as factor XIIa, IX a or Xa. Alternatively, as described by Bjoern et al. (Research Disclosure, 269 September 1986, pp. 564-565), factor VII may be activated by passing it through an ion-exchange chromatography column, such as Mono Q® (Pharmacia fine Chemicals) or the like, or by autoactivation in solution.

Factor VII equivalents may be produced by modification of wild-type Factor VII or by recombinant technology. Factor VII equivalents with altered amino acid sequence when compared to wild-type Factor VII may be produced by modifying the nucleic acid sequence encoding wild-type factor VII either by altering the amino acid codons or by removal of some of the amino acid codons in the nucleic acid encoding the natural factor VII by known means, e.g. by site-specific mutagenesis.

It will be apparent to those skilled in the art that substitutions can be made outside the regions critical to the function of the factor VIIa molecule and still result in an active polypeptide. Amino acid residues essential to the activity of the Factor VII polypeptide, and therefore preferably not subject to substitution, may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (see, e.g., Cunningham and Wells, 1989, Science 244: 1081-1085). In the latter technique, mutations are introduced at

every positively charged residue in the molecule, and the resultant mutant molecules are tested for coagulant, respectively cross-linking activity to identify amino acid residues that are critical to the activity of the molecule. Sites of substrate-enzyme interaction can also be determined by analysis of the three-dimensional structure as determined by such techniques as nuclear magnetic resonance analysis, crystallography or photoaffinity labelling (see, e.g., de Vos et al., 1992, Science 255: 306-312; Smith et al., 1992, Journal of Molecular Biology 224: 899-904; Wlodaver et al., 1992, FEBS Letters 309: 59-64).

The introduction of a mutation into the nucleic acid sequence to exchange one nucleotide for another nucleotide may be accomplished by site-directed mutagenesis using any of the methods known in the art. Particularly useful is the procedure that utilizes a super coiled, double stranded DNA vector with an insert of interest and two synthetic primers containing the desired mutation. The oligonucleotide primers, each complementary to opposite strands of the vector, extend during temperature cycling by means of Pfu DNA polymerase. On incorporation of the primers, a mutated plasmid containing staggered nicks is generated. Following temperature cycling, the product is treated with DpnI, which is specific for methylated and hemimethylated DNA to digest the parental DNA template and to select for mutation-containing synthesized DNA. Other procedures known in the art for creating, identifying and isolating variants may also be used, such as, for example, gene shuffling or phage display techniques.

Separation of polypeptides from their cell of origin may be achieved by any method known in the art, including, without limitation, removal of cell culture medium containing the desired product from an adherent cell culture; centrifugation or filtration to remove non-adherent cells; and the like.

Optionally, Factor VII polypeptides may be further purified. Purification may be achieved using any method known in the art, including, without limitation, affinity chromatography, such as, e.g., on an anti-Factor VII antibody column (see, e.g., Wakabayashi et al., J. Biol. Chem. 261:11097, 1986; and Thim et al., Biochem. 27:7785, 1988); hydrophobic interaction chromatography; ion-exchange chromatography; size exclusion chromatography; electrophoretic procedures (e.g., preparative isoelectric focusing (IEF), differential solubility (e.g., ammonium sulfate precipitation), or extraction and the like. See, generally, Scopes, Protein Purification, Springer-Verlag, New York, 1982; and Protein Purification, J.C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989. Following purification, the preparation preferably contains less than about 10% by weight, more preferably less than about 5% and most preferably less than about 1%, of non-Factor VII polypeptides derived from the host cell.

Factor VII polypeptides may be activated by proteolytic cleavage, using Factor XIIa or other proteases having trypsin-like specificity, such as, e.g., Factor IXa, kallikrein, Factor Xa, and thrombin. See, e.g., Osterud et al., Biochem. 11:2853 (1972); Thomas, U.S. Patent No. 4,456,591; and Hedner et al., J. Clin. Invest. 71:1836 (1983). Alternatively, Factor VII polypeptides may be activated by passing it through an ion-exchange chromatography column, such as Mono Q®

(Pharmacia) or the like, or by autoactivation in solution. The resulting activated Factor VII polypeptide may then be formulated and administered as described in the present application.

DESCRIPTION OF THE FIGURES

Figure 1 shows contents of aggregates and fragments after 3 months of storage at 2-8°C.

The following examples illustrate practice of the invention. These examples are for illustrative purposes only and are not intended in any way to limit the scope of the invention claimed.

EXPERIMENTAL EXAMPLES

Example 1

A. Assay Methods

Anion exchange chromatography (HPIEC) was run on a Dionex NucleoPac 100 anion exchange column at 25°C. A-buffer: 10 mM Tris, 10 mM Bis-Tris-Propane, pH 9,0. B-buffer: 10 mM Tris, 10 mM Bis-Tris-Propane, 1,4 M ammoniumacetate, pH 9,0. Elution: 0-1,5 min.: 100% A-buffer; 1,5-26,5 min.: 0-100% B-buffer. Flow: 1,0 ml/min. Detection: 280nm. Load: 80-100µg FVIIa.

Nondenaturing size exclusion chromatography was run on a Waters Protein Pak 300 SW column, 7,5x300 mm using 0,2 M ammoniumsulfat, 5% 2-propanol pH 7,0 as mobile phase. Flow rate :0,5 ml/min. Detection: 215 nm. Load: 25µg FVIIa with a 50-75 µg column load and detection at either 214 and 280 nm.

Reverse phase HPLC was run on a proprietary 4,5x250 mm butylbonded silica column with a particle size of 5µm and pore size 300Å. Column temperature: 70°C. A-buffer: 0.1% v/v trifluoracetic acid. B-buffer: 0.09% v/v trifluoracetic acid, 80% v/v acetonitrile. The column was eluted with a linear gradient from X to (X+13)% B in 30 minutes. X is adjusted so that FVIIa elutes with a retention time of approximately 26 minutes. Flow rate: 1.0 ml/min. Detection: 214 nm. Load: 25 µg FVIIa.

Example 2

Formulation Preparation

In general, aqueous FVIIa formulation samples for analysis in these experimental examples were prepared from a purified bulk solution by buffer exchange on a gel filtration column. Formulation additives were either contained in the elution buffer in their final ratios or added to the eluate. The resulting solution was sterile filtered using a sterilized membrane filter (0.2 mi-

cron pore size or equivalent) and filled into sterile glass vials, stoppered and sealed with butyl rubber stoppers and aluminum flip-off type caps.

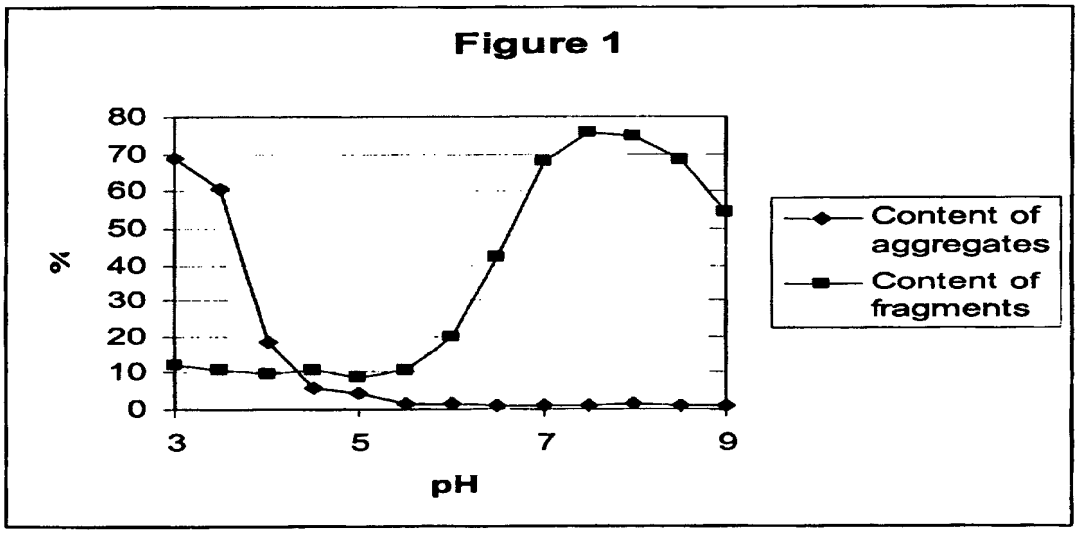
Example 3

Effect of pH on the chemical/physical stability

Vials of the rFVIIa aqueous formulation containing 1,4 mg rFVIIa/mL, 50 mM Sodium chloride, 10 mM Calcium chloride and a mixture of 10 mM glycylglycine, acetate and histidine adjusted to pH 3, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0 were incubated at either a temperature of 2-8 °C, or at elevated storage temperatures of 30 °C, and then removed at various time points and assayed for changes in pH and the chemical stability was determined by RP-HPLC and GP-HPLC.

After storage at 2-8 °C for up to three months the aqueous formulations showed insignificant changes in pH. Non-denaturing size exclusion HPLC performed on samples stored for up to three months at 2-8 °C showed no significant aggregation of the drug product at pH values ≥ 5.5 (Figure 1). RP-HPLC performed on these samples showed no significant increase in the fragmentation or oxidation of the protein in the pH range 4.5-5.5. Further, no unknown degradation products were observed.

Figure 1



The figure shows data after 3 months of storage at 2-8 degrees C. The initial content of aggregates is approximately 0.5% and the initial content of fragments is approximately 9%.

Example 4

The buffer capacity of different buffers

Vials of the rFVIIa aqueous formulation containing 1,0 mg rFVIIa/mL, 50 mM Sodium chloride, 10 mM Calcium chloride and a one of the following buffer substances in a concentration of 10 mM glycylglycine, malic acid, acetic acid, histidine, glutamic acid and citric acid were incubated at either a temperatures of 2-8.degree.C, or at elevated storage temperatures of 30.degree.C for up to 3 months. The pH was at time zero adjusted to 5.5 as this gave the least degradation products (Figure 1). The measurement of the pH in the formulation containing glycylglycine showed an increase up to 6.2 in the storage period. The other formulations showed in the same period stable values of 5.5 +/- 0.1.

Example 5

Physical Stability of Aqueous Formulations containing various detergents

Twelve different formulations were prepared. The compositions of the formulations were:

rFVIIa	0.75 mg/ml
NaCl	2.92 mg/ml
CaCl2, 2 H2O	1.47 mg/ml
Glycylglycine	1.32 mg/ml
Detergent/solubiliser	x mg/ml
pH	5.5

The concentrations of the detergents/solubilisers tested are stated in the table below.

The formulations were prepared from a liquid bulk solution of rFVIIa. Stock solutions of the detergents/solubilisers were prepared in buffers containing NaCl, CaCl2, 2 H2O, and glycylglycine in the concentrations stated above. The rFVIIa bulk and the detergent solutions were mixed, and the pH in the solutions was adjusted to 5.5. The formulations were filtered (0.2 μm) and filled in vials (1 ml solution per vial).

The appearance of the formulations was determined by visual inspection and the absorbance of the formulation at 400 nm was determined. Subsequently, the vials were shaken for 19 hours (800/min) at room temperature. After completing the shaking, the appearance and the absorbanse at 400 nm was determined. The results are listed in the table below.

Detergent type	Conc. (mg/ml)	Appearance		Absorbance (400nm)		
		Before	After	Before	After	Increase
None (reference)	-	Few part.	Very turbid	0.0085	1.4386	1.4301
Tween® 80	0.1	Very few part.	Clear, few part.	0.0044	0.0036	-0.0008

Tween® 20	0.1	Very few part.	Clear, few part.	0.0039	0.0101	0.0062
Poloxamer 188	1.0	Very few part.	Clear, few part.	0.0063	0.0027	-0.0036
Pluronic® F127	1.0	Very few part.	Clear, few part.	0.0000	0.0048	0.0048
Polyethylenglycol 400	0.1	Very few part.	Turbid	0.0076	1.5708	1.5632
Polyethylenglycol 4000	0.5	Few part.	Very turbid	0.0108	1.6624	1.6516
Brij® 35	0.1	Very few part.	Clear, few part.	0.0028	0.0015	-0.0013
Myrj® 59	0.1	Very few part.	Clear, few part.	0.0002	0.1110	0.1108
Myrj® 52	0.1	Very few part.	Clear, few part.	0.0009	0.9390	0.9381
LPCM	0.1	Very few part.	Clear, few part.	0.0026	0.0012	-0.0014
Glycerol	1.0	Very few part.	Turbid	0.0040	1.4064	1.4024

The results show that the reference (without addition of any detergent/solubiliser) becomes visually turbid when shaken and a significant increase is observed in the absorbance at 400 nm. Addition of Tween® 20 (= polysorbate 20), Tween® 80 (= polysorbate 80), Poloxamer 188, Pluronic® F127 (= poloxamer 407), Brij® 35 (= polyoxyl 23 lauryl ether), and LPCM (= α-lysophasphatidylcholine myristoyl) almost completely prevented increase in turbidy and absorbance, while a slighter increase in turbidity (as compared to the reference) was observed for Myrj® 59 (= polyoxyl 100 stearate) and Myrj® 52 (= polyoxyl 40 stearate). Glycerol, polyethylene glycol 400 or polyethylene glycol 4000 could not prevent increase in turbidity in the concentrations used in this experiment.

Example 6

Chemical stability of aqueous formulations containing methionine as antioxidant

Three different formulations were prepared. The compositions of the formulations were:

rFVIIa	0.75 mg/ml
NaCl	2.92 mg/ml
CaCl2, 2 H2O	1.47 mg/ml
Glycylglycine	1.32 mg/ml
Methionine	0 or 0.25 or 1.0 mg/ml
pH	6.5

The formulations were prepared from a liquid bulk solution of rFVIIa. The methionine was dissolved in buffers containing NaCl, $CaCl_2$, 2 H_2O, and glycylglycine in the concentrations stated above. The rFVIIa bulk and the methionine solutions were mixed, and the pH in the solutions was adjusted to 6.5. The formulations were filtered (0.2 μm) and filled in vials (1 ml solution per

vial). The vials were stored at 5°C, 25°C and 40°C. Samples were withdrawn and analysed for content of oxidized forms (by RP-HPLC) at the time point stated in the table below. The table shows the content of oxidised forms (in %).

Methionine (mg/ml)	Time zero	25°C 14 days	40°C 14 days	25°C 28 days	40°C 28 days	5°C 90 days
0 (reference)	2.4	4.4	7.5	4.4	12.8	3.1
0.25	1.7	2.4	5.3	2.8	9.9	1.9
1.0	1.6	2.3	5.0	2.6	9.6	1.3

The results show that addition of methionine slows down the oxidation rate in the formulation.

CLAIMS

1. A stable, aqueous liquid pharmaceutical formulation, comprising a factor VII polypeptide, a calcium salt, a buffer, and a tonicity modifier, wherein the formulation has a pH of from 4.0 to 7.0.

2. A formulation according to claim 1, which has a pH of from 5.0 to 6.5.

3. A formulation according to claim 1 or claim 2, further comprising an antioxidant.

4. A formulation according to claim 3, wherein the antioxidant is methionine, or a methionine analogue, or a methionine-containing peptide.

5. A formulation according to claim 4, wherein the antioxidant is L-methionine

6. A formulation according to any one of claims 1 to 5, wherein the tonicity modifier is a neutral salt; a mono-, di- or polysaccharide; a sugar alcohol; an amino acid; or a small peptide, or a mixture of at least two of said tonicity modifiers.

7. A formulation according to claim 6, wherein tonicity modifier is a neutral salt.

8. A formulation according to claim 6 or claim 7, wherein the tonicity modifier is present in a concentration of from 1 mM to 200 mM.

9. A formulation according to claim 8, wherein the concentration is 10 – 150 mM

10. A formulation according to any one of claims 1 to 9, further comprising a non-ionic surfactant.

11. A formulation according to claim 10, wherein the non-ionic surfactant is present in an amount of from 0.005 to 1% by weight.

12. A formulation according to claim 10 or claim 11, wherein the non-ionic surfactant is a polysorbate or a poloxamer or a polyoxyethylene alkyl ether

13. A formulation according to claim 12, wherein the poloxamer is poloxamer 188 or poloxamer 407, or the polysorbate is polysorbate 20 or polysorbate 80, or the polyoxyethylene alkyl ether is polyoxyl 23 lauryl ether.

14. A formulation according to any one of claims 1 to 13, wherein the buffer is selected from the list of citrate, acetate, histidine and malate, phosphate, tartaric acid, succinic acid, MES, HEPES, Imidazol, TRIS, lactate, or a mixture of at least two of said buffers.

15. A formulation according to claim 14, wherein the concentration of the buffer is from 1 mM to 50 mM

16. A formulation according to claim 15, wherein the concentration of the buffer is about 10 mM.

17. A formulation according to any one of claims 1 to 16, further comprising a stabilizer.

18. A formulation according to any one of claims 1 to 17, wherein the calcium salt is present in a concentration of 5 mM to 200 mM.

19. A formulation according to claim 18, wherein the calcium salt is present in a concentration of 10 mM to 150 mM.

20. A formulation according to claim 18 or claim 19, wherein the calcium salt is CaCl2, calcium acetate, calcium gluconate, or calcium laevulate

21. A formulation according to any one of claims 1 to 20, further comprising a preservative.

22. The formulation according to any one of claims 1 to 21, which is isotonic.

23. A formulation according to any one of claims 1 to 22, which is stable for storage for at least 6 months at 2-8°C.

24. A formulation according to any one of claims 1 to 22, which is chemically and physically stable for storage for at least 6 months at 2-8°C.

25. A formulation according to any one of claims 1 to 24, wherein the factor VII polypeptide is human factor VIIa.

26. A formulation according to claim 25, wherein the factor VII polypeptide is recombinant human factor VIIa.

27. A formulation according to any one of claims 1 to 24, wherein the factor VII polypeptide is a factor VII equivalent.

28. A formulation according to any one of claims 1 to 24, wherein the factor VII polypeptide is a factor VII sequence variant.

29. A formulation according to any one of claims 1 to 24, wherein the factor VII polypeptide is FFR-FVIIa.

30. A formulation according to claim 25, claim 26, claim 27, claim 28, or claim 29, wherein the factor VII polypeptide is present in a concentration of from 0.1 mg/ml to 10 mg/ml.

31. A formulation according to claim 30, wherein the factor VII polypeptide is present in a concentration of from 0.5 mg/ml to 5.0 mg/ml

32. A formulation according to claim 31, wherein the factor VII polypeptide is present in a concentration of from 0.6 mg/ml to 4.0 mg/ml

33. A formulation according to claim 32, wherein the factor VII polypeptide is present in a concentration of from 1.0 mg/ml to 4.0 mg/ml

34. A method for preparing a stable, liquid pharmaceutical formulation of a factor VII polypeptide, comprising preparing a formulation containing said factor VII polypeptide, a calcium salt, a buffer, and a tonicity modifier, wherein the formulation has a pH of from 4.0 to 7.0.

35. A method for preparing a formulation according to any one of claims 1 to 33, comprising preparing a formulation containing a factor VII polypeptide, a calcium salt, a buffer, and a tonicity modifier, wherein the formulation has a pH of from 4.0 to 7.0.

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